Enzyme – Directed Evolution

Solid-phase high-throughput screening of enzyme variants:
Detecting enhanced nitrilase activity

*William J. Coleman, Steven J. Robles, and Mary M. Yang**

Kairos Scientific Inc.
10225 Barnes Canyon Road #A110
San Diego CA 92121
USA
Phone (858) 626-8170
Fax (858) 626-8177
Web www.kairos-scientific.com

**Corresponding author: myang@kairos-scientific.com*

Introduction

The use of enzymes to synthesize pharmaceutical intermediates has been the focus of considerable research and development effort over the past decade, as companies seek to produce novel chemical structures while reducing the manufacturing cost and eliminating chemical waste. Enzymes and whole cells are attractive catalysts not only because of their regioselectivity and enantioselectivity, but also because they can perform biocatalytic reactions under mild conditions with fewer synthesis steps[1]. However, the stability, selectivity, and productivity of wild-type enzymes are not always optimal for chemical processing.

Development of enzyme-based processing has been hindered due to low activity on nonnatural substrates, enzyme instability, and poor or inappropriate enantioselectivity. Molecular biology tools now exist to remedy these deficiencies, including the application of directed evolution to engineer novel enzymes with enhanced properties[2-5].

Directed evolution generally consists of the following sequence of steps: (1) mutating the gene encoding the enzyme to create a large population of variants, (2) expressing the gene products in a host organism, (3) screening for the desired properties, and (4) retrieving the desired variants. These steps can be repeated until the ideal enzyme variant is produced.

Identifying enzyme variants with improved characteristics requires instrumentation and methods that provide greater throughput, information content, and flexibility to efficiently process highly complex gene libraries. Using nitrilase as an example, we describe a newly developed technology that can be used to perform these tasks.

Nitrilases and pharmaceutical synthesis

A number of key drugs or their pharmaceutical precursors contain functional groups consisting of carboxylic acids connected to chiral centers. These include, for example, the cholesterol-lowering drug Lipitor® (atorvastatin) and the analgesic Aleve® ((S)-naproxen). Methods for converting nitrile derivatives to yield chiral products using conventional chemistry have the disadvantage of requiring harsh chemicals that generate undesirable waste products. Consequently, enzyme-based synthetic routes have been developed using nitrilases to convert nitrile-containing precursors into the (R)- and (S)-enantiomers of the corresponding carboxylic acid[1,6,7] *(Figure 1)*.

Up to now, high-throughput screening of nitrilase libraries was typically performed in a liquid-phase format using standard 96- or 384-well microplates. By utilizing colored indicators or fluorogenic reagents, the reaction products can be detected by plate readers equipped with absorbance or fluorescence detectors[6]. However, microplate-based systems require robotic handling and dispensing to operate in a high-throughput mode. This type of operation requires large numbers of plates and large volumes of potentially difficult-to-synthesize or expensive substrates—which nevertheless may only facilitate screening rates of several thousand variants per day. Increasing the screening rate to 10^5–10^6 variants per instrument per day while simultaneously simplifying the sample handling and minimizing reagent usage will make it possible to screen much larger mutagenized-enzyme libraries.

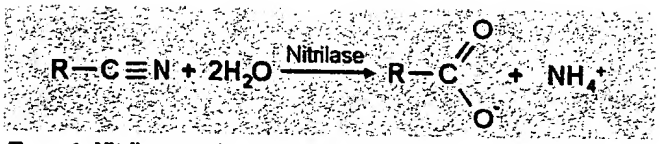

Figure 1. Nitrilase-catalyzed hydrolysis of nitriles into carboxylic acids

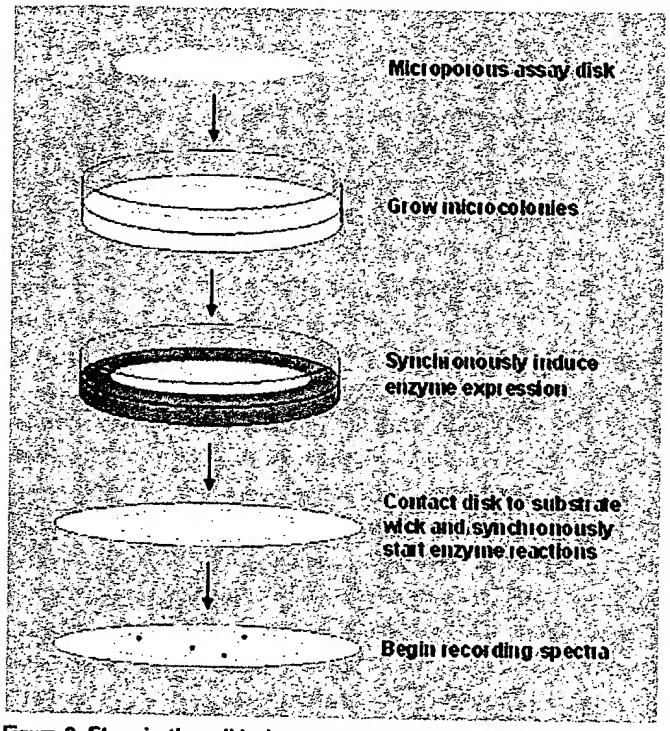

Figure 2. Steps in the solid-phase enzyme evolution assay system

Kcat technology for solid-phase enzyme screening

To achieve these enhancements, Kairos Scientific (San Diego, CA) has developed an integrated system that uses a high-density, solid-phase format in combination with digital imaging spectroscopy (DIS) to screen enzyme variants expressed in microcolonies. DIS combines image processing and optical spectroscopy so that complete spectral information can be obtained for every pixel or feature in a target image. The commercial platform for enzyme screening is known as Kcat™ Technology and includes assays, instrumentation, and computer algorithms. This technology[2,8] has been employed to engineer the properties of industrial enzymes[3,9]. Kcat Technology can be used to monitor enzymatic activity on tens of thousands of different enzyme variants simultaneously. This is accomplished by acquiring full spectral and/or kinetic information from microcolonies that are simultaneously undergoing a color-forming reaction catalyzed by the enzymes they express.

The basic solid-phase assay employs a colony-forming microbial host to express the gene(s) of interest. *Figure 2* shows an example of how the assay is performed using plasmids expressed in *E. coli*. Briefly:

1) *E. coli* cells transformed with plasmids containing the mutagenized library are randomly deposited on a circular, microporous membrane (the assay disk).

2) The assay disk is transferred to LB agar containing antibiotics, and the cells are incubated at 37°C overnight until they form microcolonies.

3) The assay disk is transferred to agar medium containing an inducer (e.g., IPTG) to synchronously induce expression of the enzyme in all the cells. (This step is optional.)

4) The assay disk is transferred to a wick containing the substrate to synchronously initiate the enzyme reaction. This is done inside the temperature-controlled Kcat device (e.g., at 37°C) to begin the measurement.

5) Images are acquired over time at the appropriate wavelength(s) to monitor the progress of the enzymatic activity simultaneously in all of the microcolonies on the assay disk. After the data is analyzed using the Kcat software, microcolonies displaying the desired activity are picked, and their corresponding plasmids are retrieved and purified.

As another demonstration of this technology, a library of random nitrilase variants was generated by error-prone PCR mutagenesis and expressed in *E. coli* microcolonies. The solid-phase assay was performed on the substrate at 37°C to detect those mutants with the highest activity based on the increase in absorbance over time (due to ammonia production). Images were acquired every 60 seconds.

Figure 3 shows a composite image of the assay disk with the reacted microcolonies and the Kcat Graphical User Interface, or GUI. The assay disk is 47 mm in diameter and contains approximately 9,000 individual microcolonies. The GUI has three interactive windows that can be used to visualize and analyze the assay data, which in this case consists of approximately 15,000 kinetic traces. The kinetics for each pixel are sorted (*Figure 4*) and displayed as thin horizontal lines in the Contour Plot Window (*Figure 3*, right). Absorbance of each pixel at a given time is color-coded from black/blue (low) to pink/white (high) according to the color scale at the bottom.

Several pixels representing the highest activity were selected from this window by clicking and dragging the mouse over this top por-

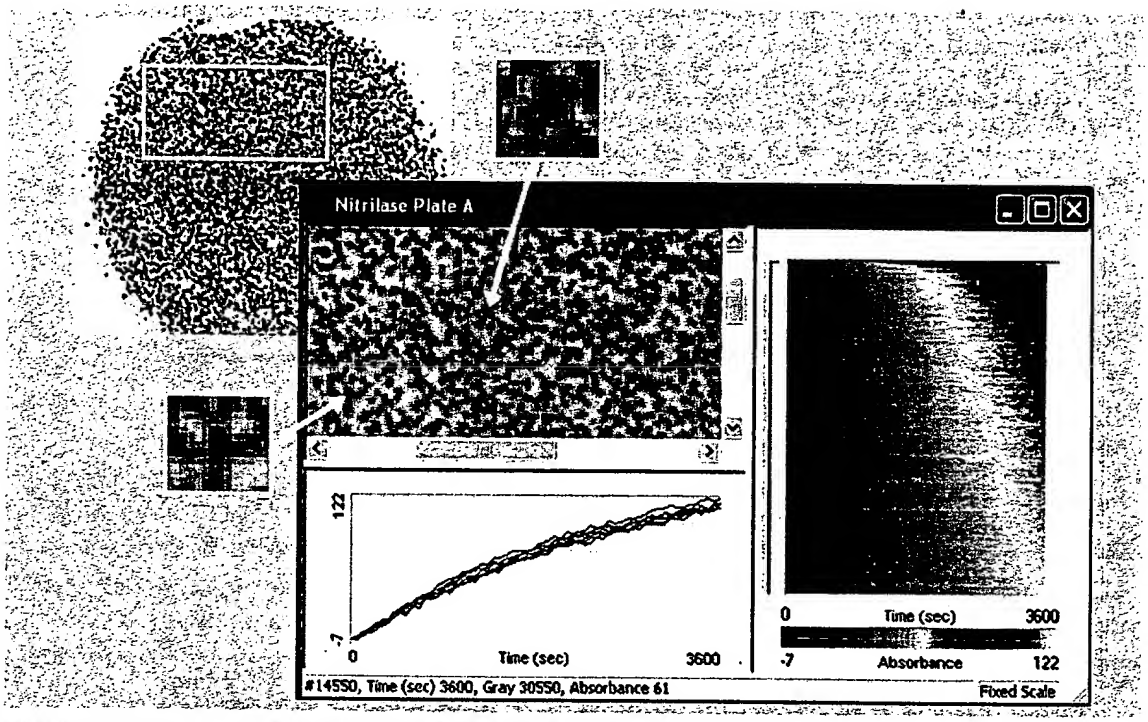

Figure 3. Screening a library of nitrilase variants using the Kcat instrument. The two most active microcolonies identified by the software are highlighted in red. Microcolonies with zero activity are not visible in the image. Assay disk diameter is 47 mm. Approximately 9,000 individual variants are on the disk.

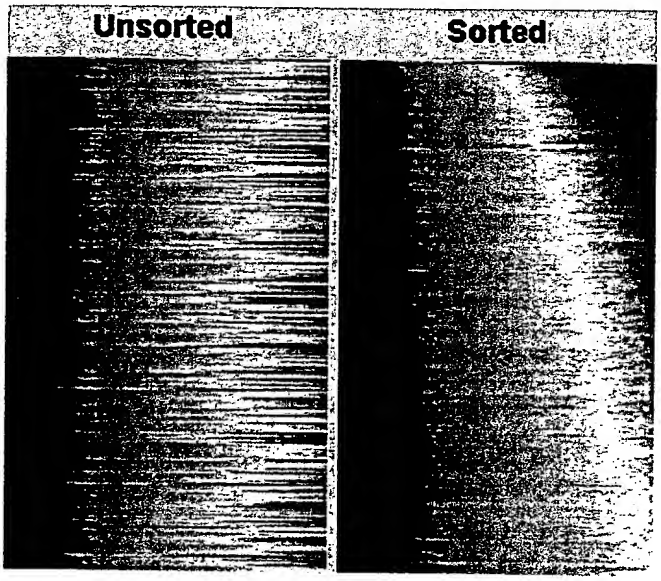

Figure 4. Contour plots displaying original unsorted kinetic data and data sorted by maximum absorbance

tion of the Contour Plot. These pixels are identified by the thin red tic mark to the left of the Contour Plot Window. The pixels selected from this window are automatically highlighted in the Image Window (top left) to identify the most active variants. The corresponding kinetics for these selected pixels are also automatically displayed in the Plot Window (bottom left).

In addition to a screening throughput of over one million variants per instrument per day, Kcat Technology provides a number of added benefits. A solid-phase format based on microcolonies has very small volume demands (ca. 50 nl). The microcolonies can be intact, lysed, or pretreated so that the enzyme is secreted. Assay design is flexible and may include coupled enzyme assays. High-molecular-weight substrates, which cannot penetrate cell walls or are not amenable to pipetting, can also be used. Due to the fact that the assay disk can be manipulated without disturbing the cells, variants can be simultaneously evolved for multiple properties by subjecting them to various physical and chemical pretreatments (heating, pH changes, etc.). The capability of acquiring full spectra over time means that the reactions can be multiplexed. This feature is convenient for comparing the enzyme activity on multiple substrates in order to change the speci-

ficity[2]. Likewise, acquisition of both spectral and time-based information enables simultaneous evolution of kinetic properties as well.

The libraries to be screened using Kcat Technology can be generated in a number of ways. Mutagenized DNA libraries can be created using error-prone PCR mutagenesis[10] to introduce random changes throughout the protein, or by a combinatorial cassette technique such as recursive ensemble mutagenesis (REM) and related methods[11-13]. The latter techniques are extremely efficient for targeting specific regions of the sequence, such as an antibody-binding site or an enzyme active site. REM and its related methods have been shown to achieve a 10-millionfold improvement in the proportion of functional and unique variants as compared to purely random combinatorial mutagenesis. Kcat screening methods can also be applied to recombinant protein libraries for optimizing protein therapeutics and to environmental or genomic libraries for discovering new genes.

Conclusion

Application of Kcat Technology to enzyme screening will make it feasible to employ much more intensive mutagenesis methods for engineering projects involving biocatalysts such as nitrilase. Simplifying the sample handling and raising the throughput will facilitate the assaying of much larger, more complex libraries to identify enzymes that are active on nonnatural substrates and that have improved stability. A user-friendly system will enable individual laboratories to create their own customized enzymes for specific applications. This in turn will facilitate the development of new synthetic processes to create enantiopure chemicals for the pharmaceutical industry.

Acknowledgement

This work was supported under NIH R44GM55470 and Kairos IR&D funds.

REFERENCES

1. Sheldon RA and van Rantwijk F. Biocatalysis for sustainable organic synthesis. *Austral J Chem* 57:281-289 (2004).

2. Bylina EJ et al. Solid-phase enzyme screening. *ASM News* 66:211-217 (2000).

3. Delagrave S et al. Application of a very high-throughput digital imaging screen to evolve the enzyme galactose oxidase. *Protein Eng* 14:261-267 (2001).

4. Powell KA et al. Directed evolution and biocatalysis. *Angew Chem Int Ed Engl* 40:3948-3959 (2001).

5. DeSantis G et al. Creation of a productive, highly enantioselective nitrilase through gene site saturation mutagenesis (GSSM). *J Am Chem Soc* 125:11476-11477 (2003).

6. Banerjee A, Kaul P, Sharma R, and Banerjee UC. A high-throughput amenable colorimetric assay for enantioselective screening of nitrilase-producing microorganisms using pH sensitive indicators. *J Biomol Screen* 8:559-65 (2003).

7. Robertson DE et al. Exploring nitrilase sequence space for enantioselective catalysis. *Appl Environ Microbiol* 70:2429-2436 (2004).

8. Coleman WJ, Robles SJ, and Yang MM. Engineering enzymes at high throughput. *Gen Eng News* 23:34, 39 (2003).

9. Delagrave S et al. Combinatorial mutagenesis algorithms, digital imaging spectroscopy, and solid-phase assays for directed evolution. In: *Enzyme Functionality*, Svendsen A (ed.), pp. 507-523, Marcel Dekker, New York (2004).

10. Leung DW, Chen E, and Goeddel DV. A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique* 1:11-15 (1989).

11. Goldman ER and Youvan DC. An algorithmically optimized combinatorial library screened by digital imaging spectroscopy. *Bio/Technology (NY)* 10:1557-1561 (1992).

12. Delagrave S and Youvan DC. Searching sequence space to engineer proteins: Exponential ensemble mutagenesis. *Bio/Technology (NY)* 11:1548-1552 (1993).

13. Delagrave S, Goldman ER, and Youvan DC. Recursive ensemble mutagenesis. *Protein Eng* 6:327-331 (1993).